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# Preliminary toxicity assessment of pharmaceutical solutions with and without ferrate treatment

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**Abstract:** An emerging challenge to the scientific community and water industries is water contamination by various micro-pollutants which are pharmaceuticals, personal care products and endocrine disrupting chemicals. The main sources of these micro pollutants are waste waters from industrial effluent and sewage treatment plants. Recent advances in the knowledge have raised concerns about their effects on the human health and ecosystems. In this study, the efficiency of ferrate to remove low concentration of two pharmaceuticals (Simvastatin and Ivermectin, 10 and 100 µg/L) was investigated. Moreover, Zebra fish animal model was employed to assess the acute toxicity before and after treatment with ferrate. Mortality, developmental changes (hatching, heart rate, total abnormalities count) and behaviour changes (swimming and activity) were recorded at all stages. Biochemical responses of Chloramphenicol acetyltransferase (CAT), Tumor necrosis factor (TNF), Interleukin 1 (IL-1) and B-cell lymphoma 2 (Bcl-2) were assessed. The study demonstrated that expression of CAT, TNF, IL-1 and Bcl-2 genes were affected after exposing zebra fish embryos to low concentration pharmaceuticals for 5 days (120 hours post fertilization). Simvastatin significantly increased the expression of anti-oxidant gene (CAT) and decreased the expression of Immune related TNF gene and apoptosis related Bcl-2 gene. Ivermectin showed significant change of expression of CAT and Bcl-2 genes. Ferrate reduced the toxicity of pharmaceuticals by partially removing them during the treatment process.

**Keywords:** Ferrate, gene study, micro-pollutants, Simvastatin, Ivermectin, toxicity, waste water treatment, zebra fish

## 1. Introduction

Pharmaceuticals, personal care products, and endocrine disrupting chemicals are categorized as micropollutants. Because of their low concentration range, from several µg/L to ng/L, and their raised concerns about adverse effects on the human health and ecosystems, micropollutant is an emerging challenge to the water industries and scientific community. The removal of micropollutants and the associated toxicity study have been attracting many researchers world widely.

Technologies like ozonation, nanofiltration, reverse osmosis and activated carbon adsorption have been widely

studied to remove pharmaceuticals in waste water treatment works. Though these technologies show the treatment efficiency for the low concentration pharmaceuticals, advanced technologies are still sought to enhance the treatment efficiency to eliminate micropollutants completely. For the toxicity study, large numbers of new compounds, which can cause negative effects on aquatic organisms, have been identified by the environmental boards [1]. It is extremely important to study the toxicity of each and every compound as some compounds may not show immediate impact when exposed but may cause a cumulative effect on the longer exposure time.

As one of alternative water treatment chemicals, ferrate has been researched in recent years to treat emerging micropollutants. Ferrate exhibits dual actions as an oxidant and coagulant and possesses high re-dox potential [2]. Early results have shown that ferrate is very effective in the oxidation of carbohydrates, nitrogen-containing pollutants and electron-rich moieties containing micropollutants [3-9].

The zebrafish (*Danio rerio*) is the tropical freshwater fish belonging to Cyprinidae family. It has been used widely in the fields of toxicology, environmental science, stem cell research, regenerative medicine, oncology, teratology, genetics, developmental biology reproductive studies and evolutionary theory studies [10-15]. The zebrafish is a very attractive model for the research because of its unique features like easy maintenance in the labs, availability of large number of eggs, easily observable and transparent large eggs; embryos development outside from their mother body, rapid development, behaviour changes, very sensitive to toxic substances, and its genome is fully sequenced for the gene and biomolecular studies.

A number of studies have reported the change in expression of oxidative stress genes (GST, GPx, GR and CAT) [16,17] due to pharmaceutical and heavy metal exposures in different animal models. Ivermectin showed the genotoxic effect by changing ChE, CAT, LDH and VTG levels [18]. Fluoxetine had a potential impact on zebrafish embryo by changing SERT and 5HT<sub>1A</sub> expression [19]. Oxytetracycline inhibited the catalase activity and reduced glutathione-S-transferase activity in adult zebrafish [20].

In this study, the efficiency of ferrate to remove Simvastatin and Ivermectin at two concentrations (10 and 100 µg/L, respectively) was investigated. And also, this study aims to assess the toxicity of the two stated pharmaceuticals in the treatment effluent by ferrate oxidation, using zebrafish animal model and gene assessment protocol involving the biochemical responses of oxidative stress genes (Chloramphenicol acetyltransferase, CAT), Tumour related gene (Tumor necrosis factor  $\alpha$ , TNF- $\alpha$ ), Inflammation-related gene (Interleukin 1, IL-1) and apoptosis-related gene (B-cell lymphoma 2, Bcl-2).

## 2. Materials and Methods

### 2.1 Test chemicals

Simvastatin ( $\geq 98\%$  GC grade), Ivermectin ( $\geq 98\%$  GC grade), Potassium ferrate, Methanol, Acetonitrile, Acridine Orange dye, Fluorescein dye, Dimethyl sulfoxide (DMSO) and qPCR kit were purchased from sigma Aldrich; SPE cartridge (200mg/6 mL, 1g/ 20mL) from Phenomenex. Deionised reagent water was generated from B114 deionizer

### 2.2 Solid-phase extraction (SPE) and UV/Vis spectroscopy measurement

Stock solution (10 mg/L) of pharmaceuticals was prepared using deionised water. The model wastewater samples (10 and 100 µg/L) were prepared from the stock solution with tap water.

Samples were extracted using strata-X, Phenomenex reversed phase cartridges. Cartridges were preconditioned with selected solvent, followed by 5 ml of deionized water. After loading samples, cartridges were washed and dried for 15 minutes under vacuum. Finally, the sample is eluted with 5 ml of selected solvent. The whole extraction process under vacuum was at a flow rate of 5-10 ml per minute [4,21].

Calibration curves for Simvastatin and Ivermectin were prepared using a selective solvent (same used for elution in SPE). Spectra scan was run between wavelength 200 and 1100 nm. Calibration was performed thrice. The strongest absorbance wavelength ( $\lambda_{\max}$ ) was determined. Using  $\lambda_{\max}$ , the absorbance of each standard sample solution (0.1-10mg/L) was determined and a calibration curve was constructed. The  $\lambda_{\max}$  for simvastatin (Sim) was 238 nm and for ivermectin (Ive) was 240 nm. The calibration equations with the linear regression for Sim is  $y=12.788x$  with correlation coefficient ( $R^2$ ) of 0.9936, and for Ive is  $y=27.029x$  with the  $R^2$  value of 0.9965, respectively, where  $y$  is the detected absorbance and  $x$  is the concentration of the pharmaceutical.

### 2.3 Ferrate performance

Ferrate was dissolved in 4M sodium hydroxide and the absorbance of resulting solution was measured at 505nm. The concentration of ferrate was detected using the

equation of  $(\text{Abs}/1150) \times \text{dilution factor} \times 1000 \times 56$  gives mg/L of Fe(VI).

The model pharmaceutical sample solutions were prepared to two concentrations, 10 & 100 µg/L. Ferrate dose (1-5 mg/L) was added and pH was adjusted to pH 4-11 using 0.1M H<sub>2</sub>SO<sub>4</sub> or 0.05 M NaOH. Jar test was performed using a six-unit stirrer (Kemiraflocculator 2000, kemwater) under the following steps: fast mixing (max rpm, 2 min) then slow mixing (40 rpm, 20 min) and finally sedimentation (60 min). The supernatant was collected and evaluated by SPE & UV/Vis spectrophotometer. By comparing the results before and after addition of ferrate, best working dose and pH were determined.

### 2.4 Fish care and maintenance:

Zebrafish eggs used in this study were obtained from the fish breeding stocks at Glasgow Caledonian University (ZEBTEC, Tecniplast). The fish system is maintained with a constant flow of conditioned water ( $28 \pm 0.5$  °C, pH 7.5), 14:10 light/dark cycle and ventilation. The fish are fed with brine shrimp twice every day. The eggs were collected from breeding tanks and examined for unfertilized and abnormal eggs under a stereomicroscope at 4 hpf. The unfertilized, abnormal eggs were discarded. The healthy eggs were bleached with chlorine to prevent infections and incubated at  $28 \pm 0.5$  °C and used for the experimental purpose [8].

#### 2.4.1 Embryotoxicity essay

The chemical treatment process was performed at 6hpf. The eggs were categorized according to the number of treatment groups (2 pharmaceuticals 10 & 100 µg/L, pharmaceuticals with ferrate [2 & 3mg/L, pH 4 & 6], pH 2 – 12 samples). Each sample group has 20 eggs and was exposed to 5ml of treatment sample solution in a 6 well plate for 120 hpf period. Each group was closely monitored throughout the treatment period and toxic endpoints were recorded and analyzed at the end of the 120hpf period

#### 2.4.2 Quantitative real-time PCR assay

The embryos after the chemical exposure at 120hpf were homogenized with 1 ml TRIzol® Reagent and incubated for 5 min at room temperature allowing complete nucleoprotein complex dissociation. Chloroform (200µL) was added to each tube, then vigorously shaken for 30 sec and allowed to stand for 3 min. At 4° C, all the tubes were centrifuged at 12000  $\times$ g for 15 min and this results in phase separation of 3 layers. The top aqueous layer (contains RNA) was separated. To this 500µL of isopropanol was added, allowed to stand for 10 min. RNA pellet was formed after centrifugation (12000  $\times$ g) for 10 min at 4° C and remaining content was discarded. RNA pellet was washed with 75% ethanol by centrifuging at 7500  $\times$ g for 10 min at 4° C. The contents were allowed to stand for evaporation of alcohol and the RNA pellet was resuspended with 25µL of RNase water, incubated for 15 minutes at 55-60 ° C and concentration were measured

using Nanodrop spectrophotometer. The extracted RNA was stored at  $-80^{\circ}\text{C}$  and was used for cDNA synthesis.

Roche Transcriptor High Fidelity cDNA Synthesis Kit was used to synthesizing cDNA from RNA according to manufacturer protocol. The concentration of synthesized cDNA was measured using Nanodrop spectrophotometer.

The gene expression was analyzed using Platinum® SYBR® Green qPCR SuperMix-UDG master mix and according to manufacturer protocol. The test was performed in duplicate with triplicate samples for each studied gene.

Biorad CFX96™ Real-Time PCR detection system was used for the assay. PCR conditions were: UDG incubation ( $50^{\circ}\text{C}$ , 2 min), Taq polymerase activation ( $95^{\circ}\text{C}$  for 2 min) and amplification (40 cycles,  $95^{\circ}\text{C}$  for

15 sec;  $55^{\circ}\text{C}$  for 30 sec and  $72^{\circ}\text{C}$  for 1 min). Fluorescence signal detection was measured at  $72^{\circ}\text{C}$ .  $2^{-\Delta\Delta\text{CT}}$  method was used to calculate the Ct based relative quantification of a candidate gene by normalizing to Ct of the reference gene, ZF18sRNA.

## 2.5 Statistical analysis

Ordinary One-way ANOVA (and Nonparametric) analysis was used to analyze the obtained data. Dunnett's multiple comparisons test was performed and 95% confidence interval and a significance level of 0.05 were adopted.

## 3 Results and Discussion

### 3.1 Performance of ferrate

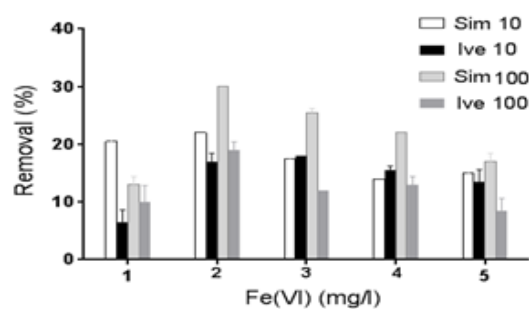
The removal efficiency of pharmaceuticals by ferrate can be seen in Figure 1. For a ferrate dose of 2mg/L as Fe and for the 100µg/L samples, the removal efficiency was 30% for simvastatin and 19% for Ivermectin, and for the same ferrate dose and 10µg/L

samples, that was 22% and 18% for Simvastatin and Ivermectin, respectively. The pH of the sample solution after addition of ferrate increased overall; from pH 7.2 to pH 12.2, depending on the ferrate doses applied (1 – 5 mg/L as Fe).

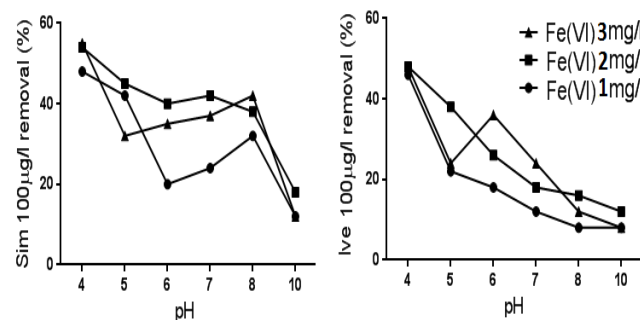
Pharmaceutical removal by ferrate was affected by the pH (Fig 2), where, the test samples had initial concentration of 100µg/L and ferrate dose employed was 1-3 mg/L. The removal efficiency of ferrate was higher at pH 4 when compared to other pH conditions for all ferrate doses.

### 3.2 Embryotoxicity study

To study the effect of pH on the resulting toxicity, the sample solution (after addition of ferrate) was adjusted to required pH (2-12) using 0.1M H<sub>2</sub>SO<sub>4</sub> or 0.05 M NaOH and tested on zebrafish eggs. Toxicity test clearly demonstrated that mortality rate is significant in case of pH 2 to 4 and 10 to 12. Though there is no significant mortality for pH 5 and 9, there is increased mortality when compared to control. (Figure 3).

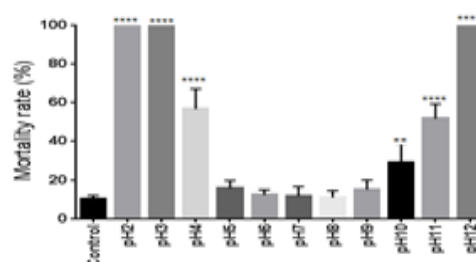


**Figure 1:** Performance of ferrate to remove Simvastatin and Ivermectin (Note: sim 10 = Simvastatin 10 µg/L; sim 100 = Simvastatin 100 µg/L and Ive 10 = Ivermectin 10 µg/L; Ive 100 = Ivermectin 100 µg/L)



**Figure 2:** Influence of pH on performance of ferrate to remove 100µg/L of Ivermectin and Simvastatin

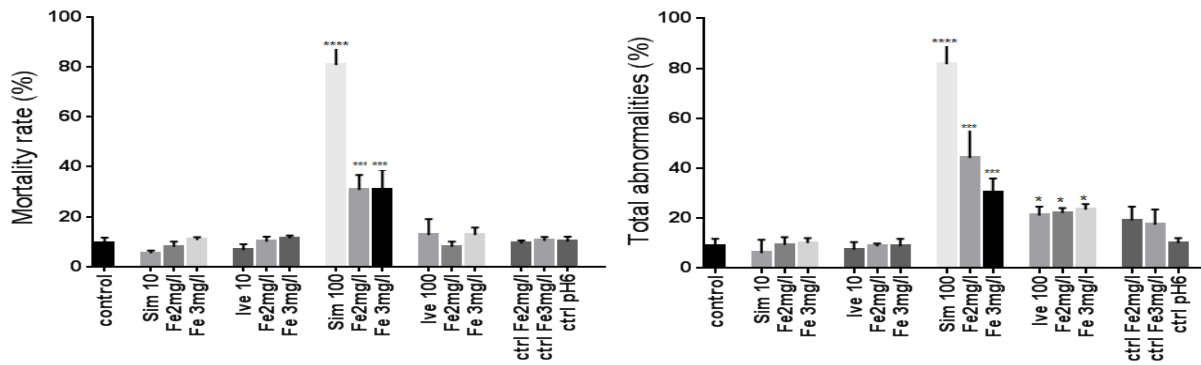
Simvastatin has clearly exhibited toxicity at concentration of 100µg/L. Mortality and total abnormalities were significantly raised to nearly 80% and there is significant decrease in hatching and heart rate when compared to the control. Simvastatin showed toxicity after ferrate treatment, suggesting incomplete removal of drug from sample solution. Ivermectin also showed increase in total abnormalities and mortality rate when compared to the control (Figures 4 and 5).



**Figure 3.** Effect of pH on zebrafish embryos mortality at 120 hpf.

Control = Zebrafish embryo water. Test samples were prepared by adjusting pH with 0.1M H<sub>2</sub>SO<sub>4</sub> or 0.05 M NaOH to zebrafish water. Results are shown in error bar as the mean ± standard deviation (SD).

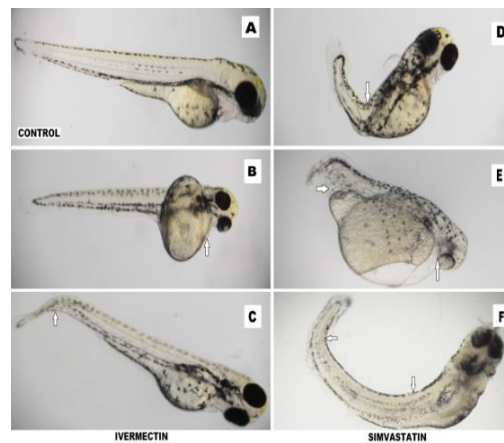
\*\*\*\* represents  $p < 0.0001$ ; \*\*\* represents  $p < 0.0002$ ; \*\* represents  $p < 0.005$



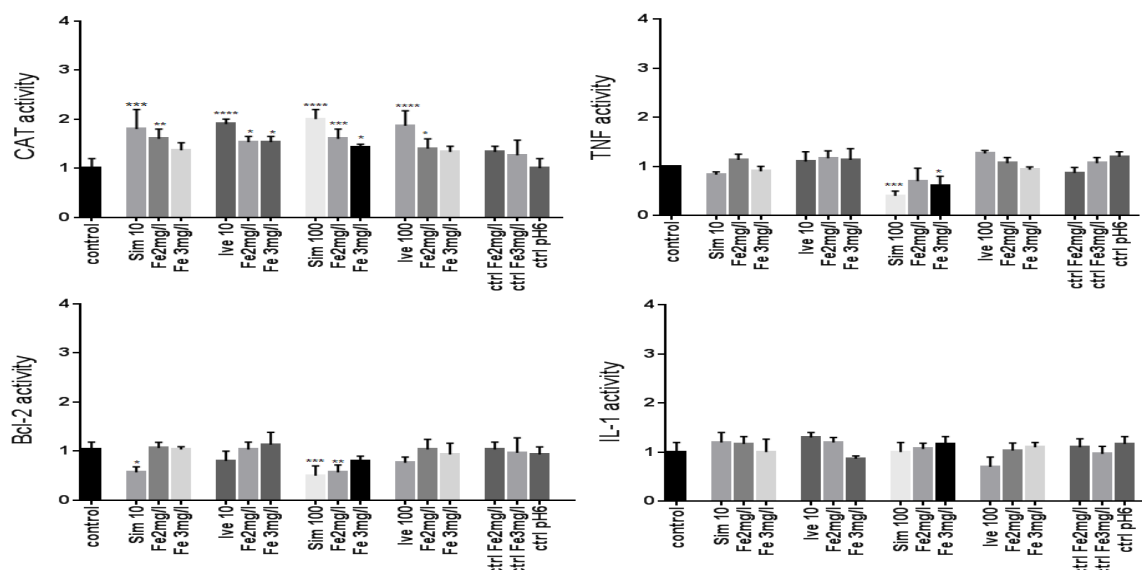
**Figure 4.** Effect of 100µg/L test samples (Simvastatin and Ivermectin) at pH6 on zebrafish embryos at 120 hpf before and after ferrate (2 and 3 mg/L) treatment.

Control = Zebrafish embryo water. Ctrl Fe 2/3 mg/L = ferrate dose without pharmaceuticals. ctrl pH6= zebrafish embryo water adjusted to pH6. Results are shown in error bar as the mean  $\pm$  standard deviation (SD).

\*\*\*\* represents  $p < 0.0001$ ; \*\*\* represents  $p < 0.0002$ ; \*\* represents  $p < 0.005$ ; \* represents  $p < 0.05$ .



**Figure 5.** Represents abnormalities in zebrafish development, left: Ivermectin and right: Simvastatin. Control A = Zebrafish embryo water; B and C= Ivermectin 100µg/L; D, E and F= Simvastatin 100 µg/L. D, E and F show abnormality in spinal cord formation; B and E shows abnormality in heart and yolk sac; C, E and F show abnormality in tail formation.



**Figure 6.** Effect of 100µg/L test samples (Simvastatin and Ivermectin) at pH6 on zebrafish embryos gene expression at 120 hpf before and after ferrate (2 and 3 mg/L) treatment.

Control = Zebrafish embryo water. Ctrl Fe 2/3 mg/l = ferrate dose without pharmaceuticals. ctrl pH6= zebrafish embryo water adjusted to pH6. Results are shown in error bar as the mean  $\pm$  standard deviation (SD).

\*\*\*\* represents  $p < 0.0001$ ; \*\*\* represents  $p < 0.0002$ ; \*\* represents  $p < 0.005$ ; \* represents  $p < 0.05$ .

### 3.3 Gene study

Simvastatin at concentration of 10 and 100  $\mu$ g/L changed the expression of oxidative stress gene (CAT), Tumour related gene (TNF), apoptosis related gene (Bcl-2) and Inflammatory related gene (IL-1). Ivermectin showed significant change in the expression of CAT gene when compared to control (Figure 6). Ferrate at dose of 2 or 3 mg/L did not show any kind of toxicity

### Conclusions

For pH 6, ferrate (VI) has shown a removal of at least 40% of Simvastatin and more than 20% of Ivermectin at a dose of 2 mg/L as Fe. Increase in dose of Ferrate (VI) didn't help to increase the removal efficiency. For pH 4, the removal of both pharmaceuticals is more than 45%. The toxicity was tested using zebra fish animal model for all pH ranges (pH 2 - 12). The results clearly indicate the toxicity was in all the cases other than pH range 5-9.

Toxicity studies clearly demonstrate that Simvastatin is very toxic even at a very low concentration such as 10 and 100  $\mu$ g/L and can also modify the expression of oxidative stress gene (CAT), Tumour related gene (TNF), apoptosis related gene (Bcl-2) and Inflammatory related gene (IL-1). Though Ivermectin is not fatal to zebrafish embryos, it produced significant number of abnormalities and also increased the expression of CAT gene, suggesting oxidative stress to the animal model during treatment procedure.

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